IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
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Serial No.: **10/030,390**

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For: **DELIVERY OF TREFOIL PEPTIDES**

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RULE 132 DECLARATION OF LOTHAR STEIDLER

I, Lothar Steidler, of The Alimentary Pharmabiotic Centre, BioSciences Building, University College Cork, Cork, Ireland, hereby declare as follows:

1. I am employed by University College Cork, Cork, Ireland.

2. I am a guest Professor at the University of Ghent, Belgium, associated with the Department of Molecular Biology.

3. I received a Ph.D degree in Biotechnology from Ghent University in Belgium.

4. I am a co-inventor of the invention claimed in the above captioned Patent Application.

5. I am a named co-inventor/applicant of WO 97/14806, which has been cited as prior art against the above captioned application.

6. I have reviewed the Office Action dated October 6, 2004, for the above captioned application, in which the Examiner rejected the claims as being unpatentable over Podolsky (WO 97/38712) and Malin (*Am.Nutr.Metabol.* 40: 137-45, 1996), in view of Steidler (WO 97/14806).

7. I supervised the following experiments, which show that a previously unknown property of a recombinant microorganism expressing a trefoil peptide *in vivo* allows the microorganism to penetrate through the mucous layer and intercalate among the cells in the intestine and thereby

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express the peptide in a way that the peptide is taken up by the cell and not degraded in the intestine:

Experiment 1: Penetration Of *L. Lactis* In Between The Cells Of The Intestinal Tissue.

A saturated overnight culture of IL-10 producing *Lactococcus lactis* MG1363[pT1mIL10] and control *L. lactis* MG1363 were concentrated 50 times in BM9. In 129 SvEv IL10-/- mice, isolated intestinal loops were inoculated with 100 µl of this suspension. Following loop inoculation, the mice were incubated for 30 minutes. The loops were dissected out subsequently and snap frozen in liquid nitrogen. Appropriate cryosections were prepared and stained and visualized with confocal laser scanning microscopy. Figure 1A shows a cross section of intestinal loop, overlapping with the luminal content (L) and tissue (T), both indicated by white bars. High power magnification (Fig. 1B) shows penetration of *L. lactis* in between the cells of the intestinal tissue. Tissues were stained with Anti-*L.lactis*, detecting *L. lactis* ; DAPI, detecting nuclei of eukaryotic cells. Cross section of intestinal loop, at the intestinal tissue shows penetration of *L. lactis* in between the cells of the intestinal tissue (Fig 1C) Tissues were stained with Anti-*L.lactis* detecting *L. lactis* ; phalloidin detecting actin as present in eukaryotic cells ; DAPI : detecting nuclei of eukaryotic cells.

Fig. 1C shows that the bacteria can penetrate in between the tissue cells in the gut, and thereby produce protein in close proximity to its receptor. That is why bacteria-produced TFF protein has a beneficial effect on inflammation in the intestine, whereas purified TFF protein simply sticks in the mucous and is ineffective (IL-10, e.g., would not stick to the mucus. For biochemical reasons IL-10 is very rapidly degraded). This mode of action could not have been foreseen at the time the invention was made. One of skill in the art would not have expected

bacteria-produced protein to be any more effective than purified protein. The benefits of the present invention are only understood with the hindsight knowledge of Applicants' disclosure.

Experiment 2: *In Vivo* Detection Of Myc Tagged TFFs Secreted By *L. Lactis*.

Ten serial inocula of 2 x 10^9 CFU of LL-pTREX1, LL-mTFF1, LL-mTFF2 or LL-mTFF3 (i.e. control *L. lactis* and *L. lactis* producing myc tagged mouse TFF1, myc tagged mouse TFF2 and myc tagged mouse TFF3 respectively, as described in Vandenbroucke *et al.* Gastroenterology, 127, 502) in 100 µL BM9 suspensions were administered to female BALB/c mice at intervals of 30 minutes A control group received ten serial inocula of 100 µL BM9. Colons were excised one hour after the last inoculation and the entire organs, including the contents, were immediately homogenized in PBS containing 1% BSA as described below Alternatively, contents were removed, tissues were rinsed with PBS and were immediately homogenized in PBS containing 1% BSA as described below. CFU values of the various *L. lactis* strains in the colon with contents averaged 5 x 10^8 (Table 2) and 5 x 10^6 in the colon tissue without contents (Table 1). The assay of Myc tagged TFF derived from *L. lactis* detected 8.7 ng mTFF1, 10.5 ng mTFF2 and 7.5 ng mTFF3 per total colon with contents (Table 1) and 0.75 ng mTFF1, 0.98 ng mTFF2 and 0.83 ng mTFF3 per colon in the colon tissue without contents (Table 2).

For quantification of Myc-tagged TFF secreted *in vivo* in colon tissue, the entire colons with contents or the colon tissue without contents were homogenized in PBS containing 1% BSA and were subsequently sonicated. Myc-tagged TFF were captured from the suspension by immobilized polyclonal rabbit anti-Myc Ab (MBL, Naka-ku Naoya, Japan), and quantified by anti-Myc biotin conjugated mouse mAb (MBL) and revealed with horseradisch peroxidase-conjugated streptavidin and reaction with TMB substrate (Pharmingen, San Diego, CA).

Table 1. *In vivo* detection of administered *L. lactis* cells and their secreted products in colon with contents

	PBS	LL-pTREX1	LL-mTFF1	LL-mTFF2	LL-mTFF3
CFU	0.20×10^1	5.24×10^9	5.72×10^8	4.04×10^8	5.28×10^8
ng MYC-tagged protein	0.00 ± 0.00	0.02 ± 0.00	8.77 ± 0.32	10.56 ± 0.26	7.51 ± 0.34

ng MYC-tagged protein represents the amount protein per colon ± SEM

Table 2. *In vivo* detection of administered *L. lactis* cells and their secreted products in the colon tissue (without contents).

	PBS	LL-pTREX1	LL-mTFF1	LL-mTFF2	LL-mTFF3
CFU	2.4×10^1	3.68×10^6	4.52×10^6	7.08×10^6	7.56×10^6
ng MYC-tagged protein	0.06 ± 0.52	0.10 ± 0.42	0.73 ± 0.50	0.98 ± 0.46	0.83 ± 0.44

ng MYC-tagged protein represents the amount protein per colon ± Standard Deviation

This experiment demonstrates that LL-TFF strains actively produced TFF *in vivo* and delivered them efficiently to the colon tissue. The bacteria concentration in the colon tissue is 100x less than the bacteria concentration in the lumen, but the TFF concentration in the tissue is only 10x less than the TFF concentration in the lumen. This indicates that the production rate of TFF per bacterium appears to be 10 fold higher for LL-TFF located in the colon tissue compared to LL-TFF located in the contents. There is a 10 fold greater amount of peptide per bacterium when the bacterium is penetrated in between the colon cells than when the bacterium is in the colon contents.

Experiment 3: Expression Of IL-10 By *L. Lactis* Penetrated In Between The Cells Of The Intestinal Tissue.

Additional studies were conducted using a microorganism that expresses IL-10. These studies show that presence of the microorganism does not lead to accumulation of the protein only in the bulk compartment of the various regions of the intestine; as would have been expected in view of WO 97/14806. Rather, they penetrate in between the intestinal cells and produce the protein there. A saturated overnight culture of IL-10 producing *Lactococcus lactis* MG1363[pT1mIL10] and control *L. lactis* MG1363 were concentrated 50 times in BM9. IL-10 knockout mice (129 SvEv IL10-/-) were inoculated two by two intra-gastricly with 100 µl of the respective bacterial suspensions. The mice were sacrificed at time points 0, 30 min, 1, 2.5 and 5 hrs after inoculation. The gastrointestinal tract was removed and indicated compartments were dissected out. The contents were collected and the intestinal tissues were washed twice with phosphorous buffered saline solution (PBS). The tissues were put in 1 ml PBS p 7.4 and homogenised. The number of colony forming units (cfu), representing the number of *L. lactis* present, was determined for both the content and the homogenized tissue of each compartment by plating on solid agar plates. The amount of mIL10 was determined in the content and in the tissue of each compartment by ELISA.

Figs. 2A and 2B show that high amounts of both *Lactococcus lactis* MG1363[pT1mIL10] and control *L. lactis* MG1363 are present in the lumen. Fig. 2C shows that mIL-10 is not detectable in any of the compartments other than the stomach. In contrast, mIL-10 is detectible in the tissue further down in the caecum and colon (see Fig. 2D). This data shows that even though mIL-10 does not survive in the lumen, the transformed bacteria produces mIL-10 in a way such that the IL-10 is able to partition into the tissue.

In a further experiment, an overnight grown culture was concentrated 50 times in BM9. Four 129 SvEv IL10-/- mice were inoculated intra-gastricly with 100 µl of this MG1363[pT1mIL10] suspension. Two mice were killed 2.5 hrs after inoculation and the remaining two mice 5 hrs after inoculation when the bacteria had reached the caecum and colon. The caecum and colon were removed. The content was isolated and the tissue was washed twice as in procedure 1. The tissue was homogenized in 1 ml PBS pH 7.4. The cfu count and the amount of mIL-10 were determined in content and the tissue. Fig. 3 shows that these compartments contain substantial amounts of bacteria, but no mIL-10, whereas the tissues contain much less bacteria but high amounts of mIL-10. As in the previous experiment, the bacteria produces mIL-10 in such a way that the mIL-10 partitions into the tissue, even though the protein does not survive in the content of the lumen.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Lothar Steidler

JANUARY 5th 2005

Date